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The use of primers for universal fingerprint ~~analyses~~ analysis

The invention relates to the use of primers or primer pairs for DNA fingerprint analysis, wherein the use of the primers or primer pairs allows to obtain fingerprints from humans as well as from animals as well as from plants as well as from microorganisms. The invention further relates to primers or primer pairs for the above-mentioned use as well as kits comprising the primers and/or primer pairs.

In the following, several prior art documents are cited, the disclosure content of which is herewith by reference incorporated into the present application.

It is generally known that the presence of polymorphic and heterogenously dispersed repetitive sequences such as microsatellites is used for genetic analysis.

It is also well-known that retrotransposons such as copia elements of Drosophila and copia-like elements in other species of the animal and plant kingdom usually are present as multiple copies in the genomes. Repetitive genomic sequences of this type were used in the example of copia-like elements in pisum (pea) for the genetic analysis of this plant species (Lee et al., Plant Mol. Biol. 15 (1990), 707-722). This method designated OFLP by the authors is based on a copia-specific primer and a second primer of a sequence of the retrotransposon flanking pea genome for PCR amplification. This made it possible to amplify pea varieties by PCR amplification of specific elements of the pea copia family and to test for polymorphisms by separation of the non-radioactively labeled PCR products and determine genetic relatedness. Also other retrotransposons, e.g. Tos1-1, Tos2-1 and Tos3-1 from rice have been used as molecular genetic markers for differentiation and identification of rice cultivars by RFLP-analysis (Fukuchi et al., Jap. J. Genetics 68 (1993), 195-204), while, however, also here it has been postulated that for other plant species their endogenous retrotransposons are isolated for use as a molecular marker. Another work (Purugganan and Wessler, Mol. Ecology 4 (1995), 265-269) uses a PCR-based method, which utilizes the variation of restriction sites for restriction enzymes in transposable elements for a fingerprint analysis. All these methods described in the

prior art have, however, in common that the described genetic markers or primers cannot be universally used in humans, plants, animals or microorganisms. It is obvious that the provision of such genetic markers or primers would offer essential advantages in many areas of modern biology or medicine. A decisive step in this direction was made by international patent application No. PCT/EP97/00442 which has not yet been published. It describes the use of primers for fingerprint analysis, wherein the primers hybridize to the copia-like element if coconut. This application for the first time provided primers or primer pairs suitable for fingerprint analysis in humans as well as animals as well as plants and microorganisms.

The problem underlying the present invention was to overcome the above-mentioned drawbacks of the published prior art and to provide methods and means which allow for a maximum degree of universal applicability of primers or genetic markers for a fingerprint analysis of species from the plant and animal kingdom as well as of humans and microorganisms. Concerning PCT/EP97/00472 further regions within the copia-like elements were to be identified which allow for a particularly advantageous derivation of the primers or further advantageous primers were to be identified.

The solution to this problem is provided by the embodiments characterized in the claims.

The published state of the art surprisingly found that the primers which hybridize with the regions characterized below of the copia-like element in coconut (Cocos nucifera L.) and which in this system permit a fingerprint analysis can also be successfully used in many other species of the animal and plant kingdom including yeast as well as in humans and even microorganisms. This finding permits to universally use said primers for fingerprint analysis in the whole animal and plant kingdom as well as in humans and microorganisms.

Thus, the present invention relates to the use of a primer or primer pair for DNA fingerprint analysis, characterized in that the primer or primer pair permits a fingerprint from humans as well as from animals as well as from plants as well as from microorganisms, wherein the primer or primer pair hybridizes with a DNA which

codes the endonuclease, the reverse transcriptase or the RNAse H of a copia or copia-like element, in particular of coconut (Cocos nucifera L.).

According to the invention, the primer/primer pair hybridizes with organisms of at lest one species of the taxonomic groups mentioned above.

In this connection, the surprising results of the present invention are achieved with arbitrary combinations of different primers of opposite polarity with the only requirement that they hybridize with the DNAs mentioned above, as well as with the use of a single primer which, due to the repetition of the copia or copia-like element, albeit in 5'→3'/3'→5' orientation of two adjacent elements and not, as represented in Figure 2B, in 5'→3'/5'→3' orientation, likewise provides the highly polymorphic fingerprints. It is self-evident that the aforementioned definition for the primers includes that they also hybridize to DNAs from other organisms as long as they contain DNA sequences which correspond to DNA sequences from the above-mentioned copia or copia-like element.

The requirements for hybridization of the primer and subsequent amplification is derivable to the person skilled in the art without inventive effort from the prior art and the following examples. Suitable conditions for the hybridization of the primers and/or the subsequent amplification can, for example, be taken from the text book Sambrook et al., "Molecular Cloning, A Laboratory Manual", CSH Press, Cold Spring Harbor, 1989 (herewith incorporated by reference) or from the following examples as well.

The term "hybridizes with a DNA which codes the endonuclease, the reverse transcriptase or the RNAse H of a copia or copia-like element, in particular of coconut (Cocos nucifera L.)" within the meaning of the present invention not only means that the primer completely and in its entire length hybridizes with said DNA. In addition, it means that it hybridizes with a DNA which overlaps with the coding DNA as defined in the above. The overlapping region comprises at least 1 nucleotide, preferably at least 5 nucleotides and particularly preferred at least 10 nucleotides.

The primers of the invention are preferably 15 to 30 nucleotides in length. The invention, however, can also be carried out with primers which are shorter or longer.

The present finding is even more surprising since as a rule the prior art started from the assumption that primers can only be used for reliable fingerprints in taxonomically narrow limits if expressive fingerprints are to be obtained.

In the prior art Rohde et al. (J. Genet. & Breed. 46 (1992), 391-394) it is described that highly repetitive sequences having homology to copia elements described in other species exist in the genome of coconut (Cocos nucifera L.), which sequences are visible as two DNA bands, 1.3 and 1.4 kilobases in length, respectively, after restriction of isolated genomic DNA with the restriction enzyme EcoRI and separation on an agarose gel. Three of these "Ecorep"-designated DNA fragments were sequenced after subcloning and sequence deviations could be determined. Attempts to use these differences for the genetic analysis of various coconut types by use of Ecorep sequences as a molecular probe in RFLP analysis or by sequence specific PCR primers were not successful (Rohde et al., J. Genet. & Breed. 46 (1992), 391-394; Rohde in: "La Recherche Europeene au Service du Cocotier - Actes du Seminaire - 8-10 septembre 1993, Montpellier". CIRAD (Collection: Colloques du CIRAD), Montpellier, pages 41-52).

Recently it was found for three coconut types that subfamilies of these 1.3 and 1.4 kilobase Ecorep sequences exist, in which these elements are clustered in the coconut genome, i.e. they represent tandem repeats, and in which usually at least one of the two expected EcoRI restriction sites at the ends of the sequence previously defined as "spacer region" is absent (Rohde et al., J. Genet. & Breed. 49 (1995), 179-186) from the previously identified elements (Rohde et al., J. Genet. & Breed. 46 (1992), 391-394). This spacer region shows high homology to the copia-like BARE-1-element from barley (Fig. 1A; Manninen and Schulman, Plant. Mol. Biol. 22 (1993), 829-846). Thus, the subfamily of copia-like sequences in the coconut genome represents tandem repeats, which display homology to the endonuclease and reverse transcriptase/RNAse H region of a copia or copia-like element (see Fig. 1B). The sequence deviations observed in the elements of the subfamily could now - in contrast to the above-described attempts for the Ecorep sequences - be used for a

genetic analysis in coconut by the appropriate PCR primers described above. This method for a genome analysis in coconut was designated as ISTR (inverse sequence tagged repeat) analysis.

It was now surprisingly found that this subfamily with its highly conserved sequence appears to be ubiquitous in the plant and animal kingdom as well as in humans and microorganisms since the use of identical ISTR-primers (see also Tables 1 and 2), which were developed on the basis of coconut sequences determined by the invention, obtained high polymorphic DNA fingerprints for other plant species as well as for animals, humans and microorganisms. In this context, not only a multitude of polymorphic markers can be discovered which segregate in the progeny ("single locus/multiple allele"-markers) but also new polymorphic markers arise (individual specific markers), which, for example, are present neither in the father nor in the mother in control cross-breedings (for example cattle, sheep) and which can be possibly ascribed to recombination events or to the amplification of specific genomic regions. Conclusively, each fingerprint is unique for the individual progeny from identical parents. In the field of human biology it could be demonstrated that this also holds true for identical twins for which several of the used ISTR primer pairs display fingerprints that are different from each other (see Fig. 8).

Thus, a preferred embodiment of the use according to the invention is characterized in that with the primer or primer pair a fingerprint is obtainable with DNAs from the entire animal and plant kingdom, comprising

(a) the animal kingdom with all its subkingdoms, preferably metazoa including the subphylums of the vertebrates, preferably the class of mammals, including in particular the family of the Hominids and the family of the Bovidae, including the species Bovis taurus and Ovis aries as well as all races and varieties which are derivable from the corresponding species;

(b) the plant kingdom with all its subkingdoms, in particular Mycobionta and Cormobionta, preferably the division of the Spermatophyta, therein preferably the class of Monocotyledonae with its families of the Areaceae and its representatives of the species Cocos nucifera or the family of Poaceae with its representatives of the species Hordeum vulgare and Zea mays, in addition most preferably the class of the Dicotyledonae with its families, for example

Solanaceae and its representatives of the species Solanum tuberosum, Nicotiana tabacum, Petunia hybrida, or e.g., the family of Brassicaceae with its representative of the species Brassica napus or the family of the Chenopodiaceae with its representative Beta vulgaris as well as all varieties and cultivars which are derivable from the corresponding species;

(c) humans; and

(d) microorganisms comprising prokaryotic microorganisms, preferably gram-positive bacteria such as, for example, lactic acid bacteria, Sarcina and corynebacteria, and gram-negative bacteria such as, for example, Neisseria and enterobacteria, and eukaryotic microorganisms comprising fungi, preferably phycomycetes such as, for example, Phytophtora, and ascomycetes such as, for example, yeast.

A particular advantage of the use according to the present invention is that fingerprints of comparable resolution and sensitivity can be visualized with DIG labeled PCR products directly in the gel without the generally used transfer of the DNA fragments onto membranes (Southern blot) well-known in the art. Thus, the present invention allows to prepare such fingerprints in a simple way (separation of the PCR fragments in a sequence gel, direct detection in the gel, computer-aided data analysis by directly scanning the sequence gel) without the use of radioactivity. Thus, a further preferred embodiment of the use according to the invention is characterized in that the DNAs to be analyzed are amplified with the primer or primer pair via PCR and subsequently separated on a gel according to the length of the PCR products.

The person skilled in the art knows how to choose the conditions for an appropriate PCR. Also a method for the separation of PCR amplified DNAs on an electrophoresis gel which preferably is a polyacrylamide gel is known from the prior art.

In a particularly preferred embodiment, the gel is a sequencing gel. The preparation of sequencing gels is also well-known in the art and, for example, described in Sambrook et al., "Molecular Cloning, A Laboratory Manual". CSH Press, Cold Spring Harbor, 1989.

In a further preferred embodiment, the use according to the invention is characterized in that in a further step a Southern blot is performed and the DNAs transferred onto the membrane are visualized by hybridization with a probe.

This embodiment is an alternative to the above-described embodiments. It requires more time and/or money and the handling of radioactivity, however, it is perfectly suitable for laboratories which have a less elaborate lab equipment, for example have no scanner with a connected computer. The performance of Southern blots as well as hybridizations with an appropriate probe are also well-known in the art and are, for example, described in Sambrook et al., loc. cit.

In a further particularly preferred embodiment of the use according to the invention, the probe is the primer or the primer pair of the invention.

Since the primers are part of the amplified DNA, the detection of the bands on the membrane used for Southern Blot can be easily performed.

In a further preferred embodiment of the use according to the invention, the primer or primer pair is labeled.

In a particularly preferred embodiment of the invention, the label is a non-radioactive label, in particular digoxigenin, biotin or a fluorescence dye, a dye or a radioactive label, in particular ^{32}P.

In particular, the labeling of the primers with digoxigenin and the dyeing of the DNA directly in the gel after amplification and gel electrophoretic separation of the DNA can be performed by all laboratories or interested breeders on the basis of a low budget equipment (PCR reaction, electrophoresis on sequence gels) and without the use of radioactivity. Storage and processing of the data is preferably performed by direct reading of the dyed and dried gel by a scanner into a computer. Furthermore, the possibility exists to develop specific primers to obtain allele specific amplification products by re-isolation of the PCR products of the sequencing gel and their re-amplification and sequencing.

In a further preferred embodiment of the invention, the primer displays the sequence as depicted in Table 2.
These primers are preferred examples of primers which were used by the inventors in previous fingerprint analyses.

Furthermore, for the use of the invention it is particularly preferred that the primer comprises a sequence which overlaps with any one of the sequences represented in table 1 or 2. The overlapping region comprises at least 1 nucleotide, preferably at least 5 nucleotides and particularly preferably at least 10 nucleotides.

The sequences of the primers that can be employed in the use of the present invention can be determined according to standard procedures, for example by sequencing the sequences adjacent to the oligonucleotide sequences in the copia or copia-like elements represented in Tables 1 and 2.

The term "overlapping sequences" comprises in the context of the invention also sequences in which one is completely contained in another. For clarification it is referred to Table 2 as well as Example 9.

In a further preferred embodiment the use of the invention is characterized in that the fingerprint analysis is used for studying biodiversity, genetic relationship, taxonomy, and, in particular, in the field of forensic medicine, breeding, protection of plant varieties, gene library management, diagnostics, population genetics and for studies on the evolution.

In addition, the invention relates to primers for the use of the present invention characterized in that the primers comprise any one of the sequences represented in Table 2 or a sequence which overlaps with any one of the sequences represented in table 1 or 2.

Moreover, the invention relates to kits comprising at least one primer and preferably at least one primer pair which hybridizes to the copia-like element of coconut depicted in Figure 2b or which have been described in the above. The primers are preferably display the sequences represented in Tables 1 and/or 2 or sequences

overlapping therewith. Said primers can be packaged in the kit of the invention in containers, for example in vessels, possibly in buffers and/or solutions. If necessary, one or more primers can be packaged within the same container. The kits of the invention can be used in manifold ways. Exemplary fields of application such as breeding have been stated in the above.

Additionally, the invention relates to the use of the primers described above for the production of kits of the invention. The production of the kits is preferably carried out according to standard procedures.

Finally, the invention relates to the use of primers which hybridize to the copia-like element of coconut depicted in Figure 2b, wherein the primer can preferably belong to one of the groups defined above, for the detection of recombination events in cross-breedings, in particular in the breeding of animals and plants.

The figures show:

Fig. 1: Region of a copia-like element Bare-1 present in the genome of barley (Figure 1A, from Manninen and Schulman, Plant. Mol. Biol. 22 (1993), 829-846) which was found as a tandem repeat copia-like sequence (Rohde et al., J. Genet. & Breed. 49 (1995), 179-186) in the genome of coconut (Cocos nucifera L.) (Figure 1B).
(A) Diagram of the copia-like BARE-1 element from barley.
ED: Endonuclease; RT: Reverse transcriptase; RH: RNAse H).
(B) Location of repetitive copia-like sequences from coconut relative to homologous sequences of the barley BARE-1 element. The hatched region characterizes the position of the recently found "spacer region" (Rohde et al., J. Genet. & Breed. 49 (1995) 179-186).

Fig. 2: Amplification of the "spacer region" between adjacent copia-like sequences in the coconut genome (A) and approximate position of previously used primers for the ISTR analysis (B).
(A) For the amplification for cloning and sequencing of the regions between two adjacent copia-like elements in the coconut the primer

pairs ISTR5/ISTR-1 and ISTR5/ISTR-2 were used. The direction of arrow heads designates the 5'→3' orientation of the oligodeoxynucleotides used.

(B) Usually, each primer is between 18 and 20 nucleotides in length and was synthesized in analogy to the sequence of the Ecorep1 element (Rohde et al., J. Genet. & Breed. 49 (1995) 179-186). The primers provided with "-" are complementary to the coding sequence of the copia element and can be combined with any primer of the "plus" series for the ISTR analysis.

Fig. 3: ISTR analysis of populations exemplified by coconut (from Rohde et al., J. Genet. & Breed. 49 (1995) 179-186).

(A) In lanes 1 to 7, single palm trees of an East African Tall (EAT) population were characterized by ISTR analysis with primer pairs ISTR5/ISTR-2 (left) and ISTR5/ISTR-1 (right), respectively. Lanes 8 and 9 are control analyses of a single Rennell Island Tall (RLT)- or Pemba Red Dwarf (PRD)-palm tree.

(B) ISTR analysis of two Malayan Yellow Dwarf (MYD)-populations from Tanzania and the Philippines with the primer pair ISTR5/ISTR-2.

Fig. 4: General application of ISTR primers in the plant kingdom.

DNA of different plant species was subjected to amplification with primers ISTR5/ISTR-2. The PCR products in the separate lanes correspond to the following plants:

1. tobacco, 2. barley, 3. potato, 4. maize, 5. snap dragon, 6. Arabidopsis, 7. rape seed, 8. Craterostigma, 9. petunia, 10. parsley, 11. sisal, 12. Milala palm, 13. Borassus palm, 14. coconut palm, 15. sugar beet, 16. Cuphea, 17. yeast.

Fig. 5: ISTR analysis of individual members of the Arecaceae (Palmae). DNAs of 17 different palm species were subjected to a standard PCR reaction with primers ISTR5/ISTR-2 and separated on a 4% PAGE gel. The PCR products in each lane correspond to the following plants:

1. Hyphaene petersiana Mart., 2. Bismarckia nobilis Hildebrandt & H. Wendl, 3. Eugeissona utilis Becc., 4. Korthalsia echinometra Becc., 5. Mauritiella aculeata (H. B. & K.) Burret, 6. Nypa fruticans Wurmb., 7. Pseudophoenix sargentii H. Wendl. ex Sarg., 8. Oraniopsis appendiculata (F.M. Bailey) J. Dransf., Irvine and N. W. Uhl, 9. Socratea exorhizza (Mart.) H. Wendl., 10. Halmoorea tripatha J. Dransf. & N. W. Uhl., 11. Cyrtostachys peekeliana Becc., 12. Deckenia nobilis H. Wendl., 13. Oncosperma tigillarium (Jack) Ridley, 14. Syagrus amara (Jacq.f.) Mart., 15. Attalea allenii H. E. Moore ex L. H. Bailey, 16. Scheelea insignis (Mart.) Karsten, 17. Asterogyne martiana (H. Wendl.) H. Wendl. ex Hemsley.

Fig. 6: ISTR analysis of barley varieties.

DNAs of 35 different barley genotypes were amplified in a standard PCR reaction with primers ISTR5/ISTR-2 and the PCR product was separated on a 4% PAGE gel. In the individual lanes the PCR products of the following plants were applied:

1. Fiction, 2. Kaskade, 3. Red, 4. Georgie, 5. Alexis, 6. Marinka, 7. Flash, 8. Portikos, 9. Aura, 10. Gimpel, 11. Prisma, 12. Gitane, 13. Gavotte, 14. Manila, 15. Pilastro, 16. Masto, 17. Torrent, 18. Thibault, 19. Onice, 20. Mette, 21. Robur, 22. Probidor, 23. Tania, 24. Mario Otter, 25. Nico, 26. Magie, 27. Vogelsanger Gold, 28. Tekto 2002, 29. Asse, 30. Calcaroides-C15 (ex Bonus), 31. calcaroides-b2 (ex Bonus), 32. calcaroides-b19 (ex Bonus), 33. Bonus, 34. Christina, 35. Nudinka.

Fig. 7: Analysis of a cattle family (A) and two sheep families (B, C).

(A) Five offspring as well as both mother and father of a cattle family were subjected to ISTR analysis with the primer pair ISTR5/ISTR-2. V: Father, M: Mother. The individual offspring are numbered. The arrow points out a marker which is not present in all individuals of the offspring.

(B, C) Analysis of two sheep families with offspring of a cross-breed between the identical father and mother M1 (B) as well as mother M2 (C). Arrows indicate segregating ISTR markers; asterisks point to

individual-specific markers, which are present neither in the parents nor in the brothers and sisters.

GSM: Marker (lower band of the triplet), which cosegrates with the male sex. V: Father. The individual offspring of the different breedings are numbered.

Fig. 8: Analysis of three human families I, II and III with different primer pairs. (A) ISTR analysis with the primer pair ISTR6/ISTR-1. (B) ISTR analysis with the primer pair ISTR6/ISTR-2. V: Father of offspring; M: Mother; SSM: sex-specific marker. The offspring are numbered. The two offspring of families I and II are identical twins.

Fig. 9: Figure 9 is a DNA analysis of grape varieties. For ISTR fingerprint analysis a PCR reaction was performed with DNA from 19 different grape genotypes with the primer pair ISTR5/ISTR-2. In the individual lanes the PCR products of the following plants were applied:

1. Sangiovese piccolo precoce, 2. Sangiovese dell'Elba, 3. Sangiovese polveroso Bonechi, 4. Colorino americano, 5. Prugnolino medio, 6. Colorino del Valdarno, 7. Morellino, 8. Brunellone, 9. Sangiovese forte, 10. Sangiovese R10, 11. Saragiolo, 12. Colorino di Pisa, 13. Prugnolino dolce, 14. Morellino di Scansano, 15. Colorino di Lucca, 16. Giacchè, 17. Tinturiér, 18. Sangiovese polveroso, 19. Prugnolo gentile.

Fig. 10: Analysis of Phytophtora palmivora isolates from the Philippines with the primer combination ISR5/ISTR-2

1: #P8704 (DRC089; Davao City, Mindanao); 2: #P8646 (DRC001; Davao Sur, Mindanao); 3: #P8652(DRC007; Davao City, Mindanao); 4: #P8650 (DRC005; Davao City, Mindanao); 5: #P8698 (DRC082; Zamboanga, Mindanao); 6: #P8684 (DRC065; De Oro City, Mindanao); 7: #P8676 (DRC053; Davao City, Mindanao); 8: #P8653 (DRC008; Davao Norte, Mindanao); 9: #P8647 (DRC002; Davao Norte, Mindanao); 10: #P8649 (DRC004; Davao Norte, Mindanao); 11: #P8662; 12: #P8663 (DRC030; Davao Norte, Mindanao); 13: #P8667 (DRC036; South Cotabato, Mindanao); 14: #P8651 (DRC006; Davao

Sur, Mindanao); 15: #P8674 (DRC047; Batangas, Luzon); 16: #P8660 (DRC025; Laguna, Luzon); 17: #P8705 (DRC090; Davao Norte, Mindanao); 18: #P8665 (DRC083; South Cotabato, Mindanao).
M: Control reaction with DNA of the MRD (Malayan Red Dwarf) coconut palm.

Fig. 11: ISTR analysis of genomic DNA with the ISTR primer pairs F6/B7 (Fig. 11A) and F21/B21 (Fig. 11B).
A. The individual lanes are DNA fingerprints of the following genomic DNAs. 1: EAT (coconut); 2: PRD (coconut); 3: SRT (coconut); 4: human being; 5: hamster; 6: rust fungus.
B. Lane 1: EAT (coconut); 2: PRD (coconut); 3: SRT (coconut); 4: human being; 5: rape; 6: barley.
The values stated above the lanes correspond to the annealing temperature selected in the standard PCR reaction.

Fig. 12: ISTR analysis of different isolates of the rust fungi Puccinia recondita f.sp. secalis with the primer combination F6/B3 (see Table 2).
DNAs of various rust fungi isolates were amplified in a standard PCR reaction with the primers F6/B3 (see Table 2) and separated on a 4% page gel.

The examples illustrate the invention.

Example 1

Detection of length polymorphisms in the coconut

For this experiment as represented in Figure 3, primer pairs ISTR5/ISTR-2 and ISTR5/ISTR-1 (see Table 1) were used. As the DNAs to be analyzed the genomic DNAs of single palm trees of populations from East African Tall (EAT) and Malayan Yellow Dwarf (MYD) as well as from a single palm tree Rennel Island Tall (RLT) and Pemba Red Dwarf (PRD) were used. The oligodeoxynucleotides employed (primers) were ^{32}P radioactively labeled at their ends via polynucleotide kinase by known means and subjected to a PCR reaction. This was conventionally performed in a volume of 20 µl and contained 1 pmol of each of the primers and 25 ng of the

genomic DNA to be amplified in 1x PCR reaction buffer (e.g. of the company GIBCO/BRL), 2.5 mM $MgCl_2$, 0.25 mM dNTP (deoxynucleoside triphosphate), and 1 unit Taq DNA polymerase. First the mixture is subjected for three minutes to 95°C for denaturation followed by 40 cycles of 95°C (30 seconds, denaturing), 45°C (30 seconds, annealing) and 72°C (2 minutes, synthesis). The reaction ends with a step at 72°C for ten minutes (synthesis), 10 µl of a dye mixture (in formamide) are added and after heating 3 µl thereof are separated on a 4% polyacrylamide sequencing gel. After separation of the glass plates, the gel is dried in a known manner on one of the sequencing plates and the separated radioactively labeled PCR products are made visible by exposure to an X-ray film.

As can be inferred from Figure 3, some of the DNA products are common to all palms but also differences in the individual palm trees of both populations are observed. This is not surprising for the "Tall" type EAT (Figure 3A), since for this coconut type cross fertilization in the field has been observed. However, surprisingly, the ISTR analysis discovers also differences in the "dwarf" palm type such as MYD which was commonly supposed to be autogamous within the populations as well as differences between the populations from Tanzania and the Philippines (Figure 3B). Such differences in dwarf populations could not be detected with previously used RFLP markers. Furthermore, it appears that the use of the primer pair ISTR5/ISTR-1 not only - as expected from the position of the ISTR-1 primer (Figure 2B) - generates PCR products which are approximately 100 base pairs shorter in length but also causes new polymorphisms. The reason for this can only be speculated on but the finding opens the possibility of using all conceivable copia-like sequences and primer combinations for the ISTR analysis based on the ascertained copia-like sequences in the coconut. Thus, this simple experiment impressively demonstrates how a single PCR amplification using the identical primer pair allows a reproducible fingerprint analysis of individual palm trees and statements on the genetic homogeneity of populations. This experimental protocol applies also to all ISTR primers and, if not indicated differently, was used for the following examples.

Table 1

Examples of oligodeoxynucleotides (ISTR primer) used for the ISTR analysis

ISTR primer sequence (5'→3')
Forward primer (SEQ ID NO.: 4)
ISTR1 AGG AGG TGA ATA CCT TAG
(SEQ ID NO.: 6)
ISTR2 AAA ATG GCA TAG TCT CTC
(SEQ ID NO.: 7)
ISTR3 GTC GAC ATG CCA TCT TTC
(SEQ ID NO.: 8)
ISTR4 TAT AGT ACC TAT TGG GTG
(SEQ ID NO.: 9)
ISTR5 ATA TAT GGA CTT AAG CAA GC
(SEQ ID NO.: 1)
ISTR6 GTA TTG TAC GTG GAT GAC ATC
(SEQ ID NO.: 13)
ISTR7 CAA CAG TGC TCC CAC TGA
(SEQ ID NO.: 12)
ISTR7' TGC TAG GAC TTT CAC AGA
(SEQ ID NO.: 2)
Backward primer
ISTR-1 TTT TCT ACT TCA TGT CTG A
(SEQ ID NO: 3)
ISTR-2 AAT AAA TCG ATC ATC GAC
(SEQ ID NO: 28)
ISTR-3 ATT CCC ATC TGC ACC AAT
(SEQ ID NO: 29)
ISTR-4 ATG TCA TCC ACG TAC AAT
(SEQ ID NO: 30)
ISTR-5 CTT CTG TGA AAG TCC TAG

Example 2

Test for a general application in plants

In order to find out whether it is generally possible to use the coconut-specific ISTR primers for the detection of DNA polymorphisms in copia-like sequences in plants, for the experiment of Example 2, the genomic DNAs of different plants were subjected to PCR reaction with the ISTR primer pair ISTR5/ISTR-2. From Figure 4 it can be seen that from tobacco to yeast DNA all DNAs used result in individual PCR products by use of the coconut-specific primers. Similar experiments were also performed with other ISTR primer combinations. This shows that sequences similar to those of the families of adjacent copia-like repetitive elements sequences described for coconut exist in lower and higher plants and are accessible for fingerprint analysis. As a

consequence, the ISTR analysis is not only applicable for a single plant species as described in Example 1 but also for the characterization of genetic diversity and the assessment of plant genetic resources either in gene libraries or by in-situ conservation.

Example 3

Test for the application within a plant family exemplified for palms (Arecaceae)

The possible application of the ISTR analysis for taxonomic studies was performed with the ISTR primer pair ISTR5/ISTR-2 in plant species of the family Arecaceae (Palmae). In this experiment, DNAs of 17 palm species (see legend of Figure 5) were amplified in a PCR reaction with the mentioned primers and PCR products were analyzed in a known manner. As can be seen in Figure 5, for each palm a different fingerprint is obtained, which permits the processing of the data via computer-aided evaluation of a corresponding matrix for the assessment of biological diversity by means of generation of dendrograms according to conventional methods. Important for the practical usability is, for example, which genetic relatedness exists, e.g. between the important oil plants of the oil and the coconut palm. Genetic markers, for example, for the feature of the thickness of the nutshell, which is important for the yield of oil, could be used in both species for breeding purposes if they are genetically highly related.

Example 4

Test for application in highly cultivated varieties illustrated for barley

Characterization of cultivated varieties via fingerprint analysis by means of the ISTR technology was tested by the example of barley varieties. Figure 6 shows a PAGE analysis of PCR products which were obtained for a total of 35 varieties and/or genotypes. The high genetic relatedness of the highly cultivated barley varieties investigated is apparent from the high number of monomorphic DNA fragments. However, even in this single analysis a total of 44 polymorphic markers could be identified which were mainly located in the upper part of the sequencing gel. These markers were grouped in a matrix and based on the matrix a dendrogram was evaluated by the UPGMA method. The fact that the variety Bonus (lane 33) could not be distinguished from calcaroides-b19 (lane 32) is not surprising since this genotype is a recessive mutant of Bonus. This, however, holds also true for the genotypes

Calcaroides-C15 (lane 30) and calcaroides-b2 (lane 31) which were generated via mutagenesis in the same genetic background. However, in this case, neutron radiation (Calcaroides-C15) and X-radiation (calcaroides-b2) were used as mutagenes, which usually lead to deletions and inversions on the chromosomal level, while calcaroides-b19 was obtained from Bonus via sodium azide treatment, which causes point mutations. Firstly, this example thus illustrates that the ISTR analysis is suitable to give indications of rearrangements of the genetic material. Secondly, the use of a single ISTR primer pair is sufficient for a fingerprint analysis of highly cultivated varieties. Thus, it can be concluded that by using further ISTR primer pairs an unambiguous variety-specific fingerprint can be obtained, which serves for biochemical characterization of the variety (protection of plant varieties).

Example 5

Test for application in animals: Evidence for segregating and newly developing markers in families

In order to test general applicability of the ISTR analysis for genetic material outside the plant kingdom, animal families were investigated in which the father was known because of controlled breeding (in-vitro fertilization). Figure 7 illustrates an ISTR analysis with the primer pair ISTR5/ISTR-2 of a cattle family (Figure 7A) and of two sheep families with identical father but two different mothers M1 (Figure 7B) and M2 (Figure 7C). Both analyses reveal that 1) coconut specific ISTR primers can also be used in the animal kingdom for fingerprint analysis and that 2) segregating markers (see arrow in Figure 7C) as well as individual specific markers (see asterisks in Figure 7) are accessible via ISTR analysis. An indication that segregating ISTR markers are capable of cosegregating with important phenotypes is evidenced by the DNA band of the prominent triplet designated as SSM (sex specific marker) in Figure 7B, C: This band is present in the father but not in the two mothers. In fact, both of the offspring of family 1 (Figure 7B) are female while family 2 (Figure 7C) has a male progeny. The fact that the parental markers are not present in all offspring (see arrow in Figure 7A) and that new markers developed (see asterisks in Figure 7), can be interpreted as an indication that the ISTR analysis is capable of discovering recombination events in cross-breedings.

Example 6

Test for application in humans: Evidence for sex and individual-specific polymorphisms

This example illustrates the application of the ISTR analysis in the field of humans. For humans, three families I, II and III were analyzed wherein both children of families I and II were homozygous (identical) twins. Since it could not be expected that ISTR primers were capable of discovering DNA polymorphisms in identical twins (highly polymorphic microsatellite primers do not display any differences, Haas, Institut für Rechtsmedizin, Universität Giessen; personal communication), 6 different ISTR primers were tested. In all 6 analyses DNA polymorphisms are visible, and two of the ISTR analyses of the primer pairs ISTR6/ISTR-1 and ISTR6/ISTR-2 are shown in Figure 8. The analysis with the primer pair ISTR6/ISTR-1 (Figure 8A) is remarkable for the multitude of polymorphic DNA bands which are individual-specific and provide an unambiguous characterization of the individual human even in the two pairs of identical twins of families I and II. This also holds true for the ISTR analysis performed with the primer pair ISTR6/ISTR-2 shown in Figure 8B, although the number of polymorphic bands is lower. Most remarkably, one DNA band is found among the new polymorphisms (SSM in Figure 8B) which is only present in the three fathers but not in the three mothers or in the five children. Actually, it is possible that, as mentioned in Example 5 for the sheep families, said DNA band concerns a sex-specific marker since all five children are female and thus a strictly sex-specific segregation within a total of 11 individuals is given.

Example 7

Detection of ISTR fingerprints for grape varieties

For this test, which is represented in Figure 9, the primer pair ISTR5/ISTR-2 (see Table 1) was used. As DNAs to be examined the genomic DNAs of 19 Vitis vinifera L. plants as well as 13 suspected "Sangiovese" genotypes and 6 "colored" ecotypes were used, the fruit of which is of importance for the intensive red coloration of the wine. It is evident from Figure 9 that a large number of polymorphous DNA fragments was obtained. Although the variability is highest in the "colored" ecotypes, ISTR analysis evidenced also a high proportion of polymorphisms in the "Sangiovese" genotypes. These differences can possibly be ascribed to the polyclonal origin of many grape cultivars. Therefore, this example, too, proves that ISTR analysis is an

efficient and sensitive method for examining the genetic diversity within ecotypes and for the identification of individual clones.

Example 8

Application of the ISTR fingerprint with microorganisms

Isolates of the fungus Phytophtora palmivora, which causes lethal diseases in coconut palms (bud rot), served as an example of the application of ISTR technology with microorganisms. In this case, a particularly difficult example of the application of a DNA marker technology was chosen, for which due to the limited genetic diversity only few polymorphisms could be expected as in all of the cases P. palmivora isolates were concerned which, moreover, were isolated exclusively in the Philippines and were there mostly locally limited (the isolates predominantly stemmed from the island Mindanao).

1 μg DNA each of 18 P. palmivora isolates from the Philippines were amplified in a standard PCR reaction with the primer combination ISTR5/ISTR-2, the products were separated according to known methods by PAGE on a 4% polyacrylamide gel and the individual lanes were made visible by autoradiography. Figure 10 shows the result of this analysis. A high number of polymorphous DNA fragments is made visible by gel analysis (Figure 10A) with a single ISTR primer combination. 30 of these bands were analysed according to known methods of the cluster analysis to obtain phenograms according to the UPGMA method (SAHN-clustering; Fig. 10B) and by PCA (principal coordinate analysis; Fig. 10C). The data obtained correspond to the classification of these isolates obtained by RAPD-DNA marker analyses.

Example 9

Specificity of ISTR analysis with overlapping primer pairs

The oligodeoxynucleotides (primers) mentioned below and stated in Table 2 were ^{32}P radioactively labeled at their ends via polynucleotide kinase by known means and subjected to a PCR reaction. This reaction was performed in a volume of 20μl according to standard methods and contained 1 pmol of each primer and 25 ng of the genomic DNA to be amplified in 1xPCR reaction buffer (e.g. from the company GIBCO/BRL), 2.5 mM $MgCl_2$, 0.25 mM dNTPs (deoxynucleoside triphosphate), and 1 unit tac-DNA-polymerase. The mixture was denaturated for 3 min. at 95°C. Then 40

cycles in total of 95°C (30 seconds, denaturation), 50°C or as stated in the legend of Fig. 11 (30 seconds, annealing) and 72°C (2 minute synthesis) were performed. A synthesis step (72°C for 10 minutes) ended the reaction, 10 µl of a conventional dye mixture (in formamide) were added and after heating 3 µl thereof were separated on a 4% polyacrylamide sequencing gel. After separation of the glass plates, the gel was dried in a known manner on one of the sequencing plates and the separated radioactively labeled PCR products are made visible by exposure to an X-ray film. This experimental protocol applies also to all ISTR primers and, if not indicated differently, was used for the following examples.

Table 2

PCR primers for the ISTR analysis with F(forward)- and B(backward) primers

Forward primers

F1	AGG AGG TGA ATA CCT TAG
F2	TTT TCT ACT TCA TGT CTG AAT
F3	AAA ATG GCA TAG TCT CTC
F4	GTC GAC ATG CCA TCT TTC
F5	TAT AGT ACC TAT TGG GTG
F6	ATA TAT GGA CTT AAG CAA GC
F7	GTA TTG TAC GTG GAT GAC ATC C
F8	CAA CAG CGC TCC CAC TGA
F9	TGC TAG GAC TTT CAC AGA
F10	CAA CAG TGC TCC CAC TGA
F11	TAA TAG TGC TCC CAT TGA TCT
F12	TTG GAC AAC CAT ATT TTG ACT
F13	ATA TGG ACT TAA GCA AGC CA
F14	ACC CTT TTC TAC TTC ATG TCT
F15	GAT CAA AAA GTT TGG TTT CAT
F16	TAG AGT TTT CCA TAC TAA ACC
F17	GCT CGG TAC CCA TAT ATG G
F18	CAT ATT GGC GTT CAT GGA G
F19	TCC ATG AAA GAC CTA GGT GA
F20	AGT ATG GAA AAC TCT AAG AGG
F21	ATA TAT GGA CTT AAG CAA GCA TCT CGG AGC

Backward primers

B1 (SEQ ID NO 25) TTT TCT ACT TCA TGT CTG AAT
B2 (SEQ ID NO: 26) AAT AAA TCG ATC ATC GAC TC
B3 (SEQ ID NO 27) GGA TAT CCT ATG AAT CAA GC
B4 (SEQ ID NO: 28) ATT CCC ATC TGC ACC AAT
B5 (SEQ ID NO 29) ATG TCA TCC ACG TAC AAT
B6 (SEQ ID NO 30) CTT CTG TGA AAG TCC TAG
B7 (SEQ ID NO 31) AAT CGT GTA TCT TCA AAA AAG
B8 (SEQ ID NO 32) ATA TAT GGA CTT AAG CAA GCA
B9 (SEQ ID NO 33) GGA ATA TCA TTC CCA ATA AG
B10 (SEQ ID NO 34) CCT CCT TAT TGG GAA TGA TAT
B11 (SEQ ID NO 35) GAA ACG AGT GTT CCA GTT C
B12 (SEQ ID NO 36) GAC CCT TTT GAA AAC ACA TG
B13 (SEQ ID NO 37) TCT TGG AGT TGG AAC ACT C
B14 (SEQ ID NO 38) GTT TCA ATG ATG TGA TCA AAA A
B15 (SEQ ID NO 39) GGG TAT TAA TCC CCT CCT AG
B16 (SEQ ID NO 40) AAA CCT AGC GGC TAT TCC AT
B17 (SEQ ID NO 41) GGC TAC AAT AGC ATG CAA TG
B18 (SEQ ID NO 42) CAG AGT TGA TAT CTG ATA TCG
B19 (SEQ ID NO 43) CCT CTA TAT CCT TTG AAA TAG
B20 (SEQ ID NO: 44) CAC ATT GTG ATC TTC TAT AAT
B21 (SEQ ID NO: 45) AAT AAA TCG ATC ATC GAC TCT AAA GGA CCT

In the below-mentioned example of the temperature dependence of the PCR amplification during ISTR analysis 2 primer pairs were used which i) are overlapping (F6 and F2; B7 and B21; see Table 2) and ii) have a different length:

i) overlapping primers
A) ISTR primer pair F6(20mer)/B7(21mer)
B) ISTR primer pair F21(30mer)/B21(30mer)

The total sequence of primers F6 and B7 is contained in the primers F21 and B21, respectively. The results shown in Fig. 11A and B show that also overlapping primers can be used for ISTR analysis and lead to DNA fingerprints that are different from each other (lanes 1-3, respectively).

ii) Temperature-dependence of the ISTR analysis

The specificity of ISTR primers as compared to the so-called "arbitrary primers" (arbitrarily priming oligodeoxynucleotides), as they have been described by J. Welsh and M. McClelland in "Fingerprinting genomes using PCR with arbitrary primers", Nucleic Acids Res., 18:7213-7218 (1990), has been shown by means of the temperature dependence of the PCR reaction. Whereas the primers described by Welsh and McClelland themselves up to a length of 34 nucleotides revealed no PCR amplification at 52°C annealing temperature (op. cit. p. 7215), ISTR analysis with 30mer-ISTR-primers even at a temperature of 72°C showed discrete and reproducible fingerprints in the homologue (coconut) as well as in the heterologue system (human, rape, barley) (Fig. 11B). As could not have been expected differently on the basis of the teaching of the present application in the case of the length of the selected primers in the ISTR primer pair F6(20mer)/B7(21mer), at an annealing temperature of 55°C a good amplification could be obtained, which was not the case however, at 60°C (Fig. 11A).

Example 10

Detection of ISTR fingerprints in different isolates of the rust fungus Puccinia recondita f. sp. secalis

Different isolates of the rust fungus Puccinia recondita f. sp. secalis served as further example of the application of ISTR technology with microorganisms. In this trial represented in Fig. 12, DNA was isolated from single-pustule isolates of the rust fungus and the primer pair F6/B3 (see Table 2) was used. The PCR reaction was carried out as described in Example 8 and the products were separated according to known means by PAGE in a 4% polyacrylamide gel and the individual bands were made visible by autoradiography. As shown in Fig. 12, by a single ISTR primer combination a high number of polymorphous DNA fragments is again reproduced by which the different rust fungus isolates can be determined. Thus, this experiment represents further evidence for the applicability of the ISTR technology of the invention in the case of microorganisms.